# DEVELOPMENT OF A RAT DIISOPROPYLFLUOROPHOSPHATE-INDUCED SEIZURE/STATUS EPILEPTICUS MODEL FOR SCREENING OF NEUROPROTECTANTS FOLLOWING EXPOSURE TO CHEMICAL WARFARE AGENTS

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## ABSTRACT

We have developed a seizure/s*tatus epilepticus* (SE) rat model using diisopropylfluorophosphate (DFP), an organophosphate (OP) surrogate for chemical warfare nerve agents (CWNA) soman and sarin. Rats were surgically implanted with a radiotelemetry probe that records EEG, ECG, body temperature, and physical activity. After 1 week they were exposed to various concentrations of DFP (1.5-4.0 mg/kg, sc) in the presence of pyridostigmine bromide, 2-PAM and atropine to produce more than 24 h seizure/SE. The successful model involves administration of pyridostigmine bromide (0.026 mg/kg, im) 30 minutes prior to DFP (4.0 mg/kg) followed by a mixture of atropine (0.2 mg/kg) and 2-PAM (25 mg/kg, im). Neuropathology data in the DFP model showed maximum and more severe damage in the amygdala, similar to CWNA treated animals. This animal model of seizure/SE can be used for evaluation of neuroprotectants and recombinant bioscavengers, as well as studying the molecular mechanism of neuronal death following exposure to chemical warfare agents.

## INTRODUCTION

Exposure to organophosphate (OP) cholinesterase inhibiting chemical warfare nerve agents (CWNAs) perturbs cholinergic neurotransmitter function and subsequently glutamatergic function. Hypercholinergy leads to neurodegenerative conditions manifested by uncontrolled seizures/*SE* that is potentially life threatening if not properly treated. The sequence of events following OP intoxication that leads to seizure/*SE* causing permanent neuropathological damage, abnormal physiological function, neurobehavioral deficits, and potentially death is not clearly known. This signifies a need for a more suitable model to study these events.

Current emergency treatment of acute OP poisoning, consisting of combined administration of an AChE re-activator (an oxime), a muscarinic ACh receptor antagonist (e.g. atropine), and an anticonvulsant (e.g. diazepam) is not general and does not prevent neuronal brain damage and the resulting incapacitation (1-7). Therefore, an animal model of seizure/SE following exposure to OP is also required to identify improved and more generic therapies for sufficient protection against OP poisoning.

Diisopropylfluorophosphate (DFP) is a prototypical OP, irreversibly inhibiting AChE in the central nervous system, blood, and other tissues following exposure (8-11). The subsequent accumulation of ACh at cholinergic synapses following OP exposure is responsible for the spectrum of toxic symptoms of OP poisoning, which vary depending on the dose, from subclinical toxicity to convulsions and death. Although DFP models of convulsions, seizure and *SE* are being used for neuroprotective drug development, most of these studies are based on evaluating seizure with behavioral studies (12-15). Even though peripheral motor convulsions occur during seizure, it cannot be deduced that it is because of the CNS depolarization and toxicity. In addition, in many of these studies the animals died few hours after DFP administration and the results are very intriguing. A

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dependable animal model capable of exhibiting seizure/SE by cortical recordings for more than 24 h is important for understanding the mechanisms of pathology and designing the measures for protection against OP exposure. For the evaluation of neuroprotectants against CWNA, ideally the model should be very similar to chemical warfare nerve agents in the kinetics, neurophysiology and neuropathology

The goal of the present study was to develop a seizure/SE model using DFP in rats and to investigate the resulting neuropathology. The basic model described herein for DFP showed similarity to chemical warfare nerve agent exposure (16) and should be applicable to other species. It will be useful in the understanding of the mechanism of neuropathology following exposure to OPs, as well as the screening of prophylactic and post exposure medical counter measurements.

## MATERIALS AND METHODS

*Animals:* All animal experiments were performed in accordance with principles stated in the Guide for Care and Use of Laboratory Animals and Animal Welfare Act. Adult male Sprague-Dawley rats (225-250 gm, Charles River laboratories, MA) were housed under standard conditions with 12 h light/dark cycle and food and water available *ad libitum*. The rats were quarantined for 1 week before surgical implantation of the radiotelemetry probes.

*Surgical implantation of the radiotelemetry probes:* The radiotelemetry probe FL-50 EET and the radiotelemetry system were purchased from Data Sciences International (St. Paul, MN).The probes were sterilized and handled according to the instructions of the manufacturer. One week after their arrival, the rats were anesthetized by isoflurane (2% isoflurane and 1.5% oxygen) and placed on a stereotax for surgery and implantation of the probes. After administration of Bupivacaine analgesic, a small incision was made along the midline of the dorsal side 3 inches cranial to the base of the tail. The two cortical electrodes and control electrode were tunneled subcutaneously to the upper surface of the skull in the presence of local anesthetic Lidocaine. The two cortical electrodes were implanted on 1 mm circumference holes, each made 3 mm on either side of the center and 3 mm above the bregma. The reference electrode was placed on the right side, 3 mm from the center and 3 mm above the sutura sagittalis. The electrodes were immobilized using screws and dental acrylic. Electrodes for ECG recordings were tunneled and placed to the right pectoral muscle and to the lower side of the xiphoid process. The body of the telemetry probe was placed on the dorsal left quadrant of the animals and sutured in place. The rats were administered with Bupivacaine post surgically at 6 to12 h interval for 24 h.

*DFP-induced seizure/SE:* In initial experiments involving lithium, seven days after the surgery, the rats received 5 mEq/kg, ip, lithium chloride (Sigma Chemical Co, St Louis, MO). On the following day, the rats housed in individual cages were placed on radiotelemetry receiver and the basal EEG, ECG, temperature and activity were recorded for 30 min. DFP induced seizure/*SE* was developed by employing several experimental conditions and drugs that are described in detail in the results. The successful model involves, recording of the basal activity for 30 min and then administration of 0.26 mg/kg, im, pyridostigmine bromide. Thirty minutes later, freshly diluted DFP in ice-cold saline (4 mg/kg, sc) was administered, followed by a mixture of atropine (0.2 mg/kg) and 2-PAM at 31 min. DFP was always kept on ice, and the dilution was done using a Hamilton syringe (Australia). Also the drugs 2-PAM, atropine, and PB need to be prepared freshly once in 2-3 months since the activity may go down during storage. The animals were then placed on the receiver, and the data was continuously monitored and recorded for 24 h. The DFP model of seizure/SE works equally well in the absence of lithium chloride pre-administration.

*Functional observation battery tests:* Functional observation battery tests consist of 25 measures of sensorimotor and autonomic functions (17-19). The measurements were made first with the animals in the cage. Animal posture, palpebral closure, and presence or absences of convulsions were scored. If convulsions were present, they were further categorized. Following observation in the cage, the animal was removed and briefly held in the hand. Ease of removal and handling, skin and fur abnormalities, lacrimation and nose secretion was recorded. Reflex testing consisted of the rat's

response to the frontal approach of a blunt object, a touch of an object to the posterior flank, and an auditory click stimulus. Reactivity to a pinch on the tail and the ability of the pupil to contract were also assessed.

*Survivability:* The overall health and survivability of the rats within the 24 h duration of the experiments were also recorded to evaluate the protective effects of the adenosine receptor agonists.

*Blood and Brain AChE assay:* At the end of the study, the animals were deeply anesthetized with 75 mg/kg pentobarbital. Blood was collected by cardiac puncture. The blood was immediately frozen for future whole blood cholinesterase assays. Blood AChE is measured (WRAIR-microassay) spectrophotometrically at 410 nm by the method of Ellman et al (20) using acetylthiocholine iodide as the substrate. Since there is an excellent correlation between brain and spinal cord AChE activity (21), the spinal cord was obtained. The cervical segment was homogenized in the presence of Triton X-100 or T-PER (Sigma Chemical Co.) in ice and centrifuged, and the total AChE activity was measured by WRAIR assay. Protein concentration was determined by Lowry's method or by BCA protein assay reagent (Pierce Chemical Co, Rockford, IL).

*Neuropathology:* Animals surviving 24 h were deeply anesthetized with 75 mg/kg pentobarbital, euthanized by exsanguinations, and transcardially perfused with 4% formadehyde. In animals, where the spinal cord sample was used for AChE assay The brain was removed and fixed with 4% formaldehyde. Serial 2 mm sections were cut using a slicing chamber, and the anteroposterior level of the sections were selected according to steriotaxic coordinates of the rat brain atlas of Paxinos and Watson (-3.0 and -4.0 from Bregma reference). Blocks will be sectioned at 5 μm and stained by hematoxylin and eosin (HE). Grades are based on histologic analysis of H&E-stained brain sections, using the criteria of McDonough, et al, (22;23) (16) with slight modifications, as follows: 0 – no neuronal necrosis, 1 – necrosis of 1-10% of neurons, 2 – necrosis of 11-25% of neurons, 3 – necrosis of 26-50% of neurons, 4 – necrosis of > 50% of neurons.

Neuronal damage was quantitated for each brain and for each staining method using 26 high magnification fields representing 4 regions of the hippocampus to examine neuronal neuronal damage. Thirteen fields were from each half of the brain, representing 3 fields of CA1, 3 fields of CA3, 5 fields of the dentate gyrus, and 2 fields of the polymorphic layer (hilus). Each field was photographed and the number of degenerate or dead neurons for each field was determined in a blind fashion through visual examination of photomicrographs by a board-certified veterinary pathologist. Based on previous studies, damaged neurons demonstrated by HE are expected to exhibit hypereosinophilic cytoplasm and hyperchromatic nuclei or, less frequently, to be shrunken and have pyknotic nuclei. All raw data was entered into an Excel spread sheet. The mean number of damaged neurons for each of the 4 regions of hippocampus will be determined and other statistical analyses will be done to compare experimental groups.

*Data analysis:* The experimental data was analyzed using Dataquest ART 2.3 (DSI International) and plotted over time. EEG data was analyzed using waveform analysis, and the latency of seizure termination was determined based on the time required to reduce the amplitude of seizure spikes $\leq 2$ standard deviation of basal EEG amplitude recorded at the beginning of each experiment. Latency period also corresponds to the time period where there was no further seizure development $\geq 10$ sec. A power spectrum of EEG data was also performed using Dataquest ART2.3. Heart rate was calculated directly from the ECG recordings using Dataquest ART 2.3. Physical activity is shown as spikes in Dataquest ART and analyzed as histograms. The body temperature was directly plotted over time in Celsius.

## RESULTS

*Lithium-DFP induced seizure following preadministration of methyl atropine*: We first attempted to develop DFP induced rat seizure/*SE* model by pre-administration of lithium and methyl atropine to prevent peripheral effects and improve the survivability following DFP. Methyl atropine does not cross the blood brain barrier and therefore will not affect the CNS seizure/SE in DFP treated animals. After one week of recovery following surgery, animals were administered with LiCl (5 mEq), im.

Twenty-four hours later the animals were randomly placed on radiotelemetry receivers, and base line EEG were recorded for 30 min before administration of methyl atropine (1 mg/kg, sc). After 30 min, DFP varying from 1.3 to 2.5 mg/kg, sc was administered in 50 µl volume. Although the animals administered with DFP 1.3 mg/kg, sc, showed strong motor convulsions, they were unable to produce any CNS seizure (1A).

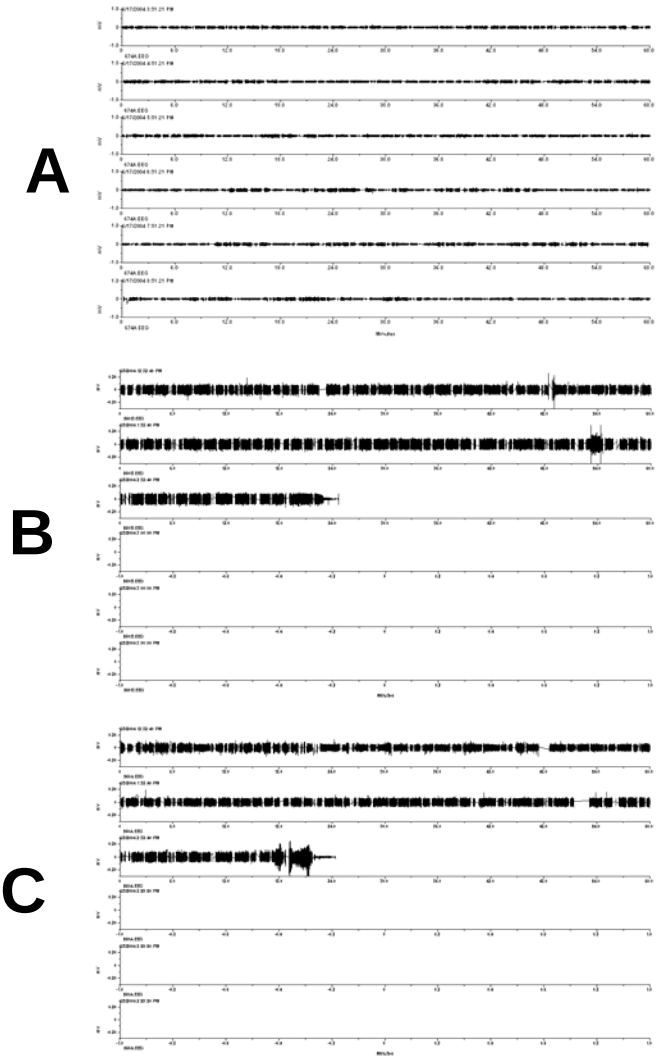


**Figure 1: Administration of DFP in a larger volume is required for the production seizure/SE.** **A**. Rats implanted with telemetry probes were administered with LiCl (5 mEq/kg, im). After 21 h baseline EEG was recorded for 30 min and then administered with saline and the EEG was recorded. **B**. Rats were administered methyl atropine (1 mg/kg) followed by DFP (2.5 mg/kg) freshly diluted in 50 ml of phosphate buffered saline. EEG was recorded continuously. The Y axis range was -1 to +1. Note that this rat died without producing any seizure. Each line represents 1 h of EEG recordings. **C**. A representative EEG recording of rats administered methyl atropine (1 mg/kg) followed by DFP (2.5 mg/kg) freshly diluted in 300 ml of phosphate buffered saline. The Y axis range was -1 to +1. Note that this rat produced a brief period of seizure represented by high amplitude spikes before dying. Each line represents 1 h of EEG recordings.

Increasing the dose of DFP to 2.0 mg/kg, sc, induced seizure in some animals. However, all the

animals that had seizure died within 10-20 min (Fig 1B). Further increase in the dose of DFP to 2.5 mg/kg produced strong seizure in all the animals in the group, but the animals died within 10-20 min (Fig. 1B). Administration of a second dose of methyl atropine 1 mg/kg, sc at the first sign of toxicity after DFP administration was unable to reduce the mortality rate. Thus, administration of DFP in the presence of methyl atropine produces short time seizure and no *SE*.

*Administration of DFP in larger volume induces seizure and SE:* Next we administered DFP 2.5 mg/kg, sc in 250 µl volume 30 min after methyl atropine (1 mg/kg, sc). Interestingly, these rats showed more defined seizure that continued as *SE* for 60 min. At around 60 min all the animals died abruptly, most likely due to cardiovascular failure (Fig. 1C). This data suggests that a larger volume of DFP is essential to induce seizure that can become SE in rats. However, once again administration of a second dose of methyl atropine was unable to improve the survivability.

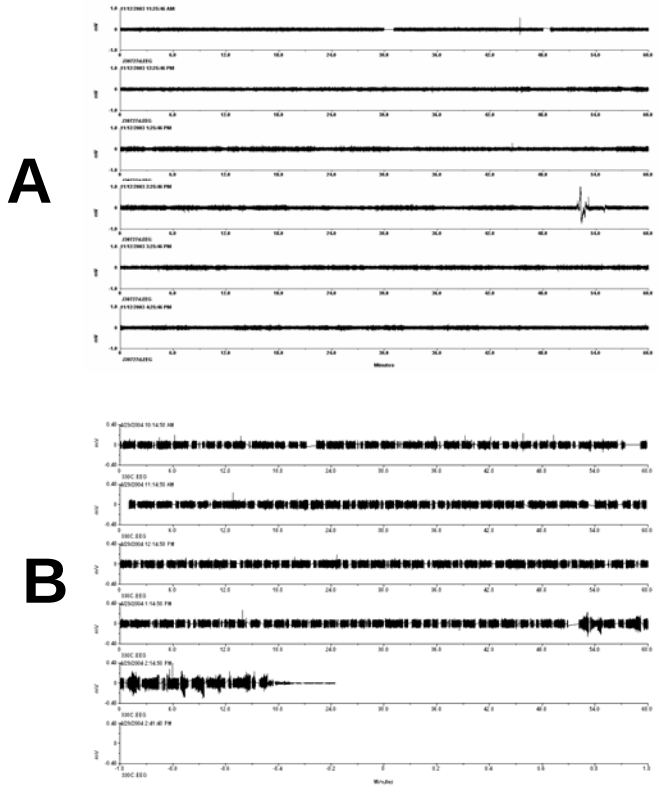


**Figure 2: Treatment with PB 30 min before DFP followed by atropine 2mg/kg and 2-PAM 25 mg/kg 1 min later increase the survivability of animal undergoing seizure/SE to 30 min. A**. Rats implanted with telemetry probes were administered LiCl (5 mEq/kg) . After 21 h, pyridostigmine bromide (0.026 mg/kg, im) followed by 300 ml saline and a mixture of atropine and 2-PAM (2 mg/kg and 25 mg/kg, im) was injected and the EEG was recorded. **B**. Rats were administered pyridostigmine bromide (0.026 mg/kg, im) followed by DFP (14 mg/kg, sc) freshly diluted in 300 ml phosphate buffered saline. One minute later a mixture of atropine and 2-PAM (2 mg/kg and 25 mg/kg, im) was injected, and the EEG was recorded continuously. The Y axis range was -1 to +1. Each line represents 1 h of EEG recordings. Note that this rat produced seizure/SE and died after 30 h.

*Lithium-DFP induced seizure/SE by administration of pyridostigmine bromide, atropine and 2 PAM:* The chemical warfare nerve agent induced seizure/SE model in rats and guinea pigs have been developed by pre administration of 0.026 mg/kg pyridostimine bromide (PB) followed by CWNA and 1 min later a mixture of atropine sulfate (2mg/kg) and 2-PAM (25 mg/kg) (16;24;25). The purpose of

the PB is to reversibly inhibit AChE and thereby temporarily shield the AChE from being inactivated by the organophosphate. Atropine is a muscarinic antagonist, and 2-PAM is an oxime that regenerates the inactivated AChE that is not aged. We have used similar conditions to improve the survivability. Our data shows that under these conditions, the amount of DFP required to induce seizure/SE was increased from 2.5 mg/kg to 10-20 mg/kg, sc (Fig 2). However, under these conditions two-thirds of the animals that received 8 mg/kg DFP did not undergo seizure/SE. Of the one-third of the animals that received 20 mg/kg DFP, all had seizure but died shortly after. Although these drug treatments did not improve the survivability of animals undergoing seizure/SE, at least we were able to increase the survivability time for 40-60 min.

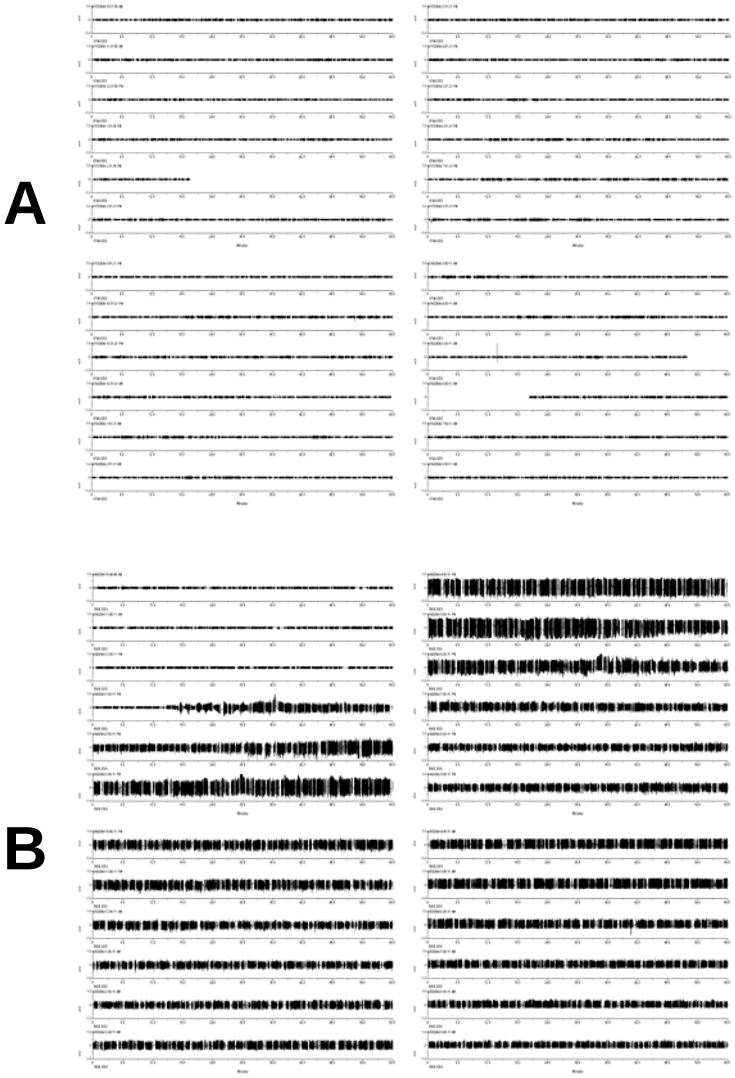


**Figure 3: Newly developed rat DFP model of seizure/SE.** Rats implanted with telemetry probes were administered with LiCl (5 mEg/kg, im). After 21 h base line EEG was recorded and the animal was administered with pyridostigmine bromide (0.026 mg/kg, im) followed by 300 ml saline and a mixture of atropine and 2-PAM (0.02 mg/kg and 25 mg/kg, im) was injected and the EEG was recorded. **B**. Rats were administered pyridostigmine bromide (0.026 mg/kg, im) followed by DFP (4 mg/kg, sc) freshly diluted in 300 ml phosphate buffered saline. One minute later a mixture of atropine and 2-PAM (0.2 mg/kg and 25 mg/kg, im) was injected, and the EEG was recorded continuously. The Y axis range was -1 to +1. Each line represents 1 h of EEG

*Administration of lower dose of atropine improves the animal survivability following lithium-DFP induced seizure/SE:* To improve the survivability of animals in the DFP model of seizure/SE we evaluated the effectiveness of reducing the dose of atropine. Animals were administered LiCl 5 mEq/kg, im. Twenty four hours later the animals were injected with 0.026 mg/kg PB, followed by various doses of DFP (3.5 or 4 mg/kg, sc) after 30 min and a mixture of atropine (0.2mg/kg) and 2-PAM (25 mg/kg, im) after 1 additional minute. We found that when the dose of atropine was reduced to 0.2 mg/kg, all the animals that produced seizure/SE following administration of 3.5 - 4.0 mg/kg DFP survived more than 24 h (Fig 3). Although 3.5 mg/kg DFP was sufficient to produce reproducible seizure/SE in most of the animals under these conditions, a dose of 4.0 mg/kg, sc, is required to produce seizure/SE in 100% of the animals. At 4.0 mg/kg DFP the animals also showed reproducible seizure with very similar kinetics of seizure/SE induction among the animals in a batch.

*Lithium is not essential for DFP induced seizure/SE:* To determine whether pre- administration of LiCl is necessary for the DFP induced seizure/SE we omitted the lithium administration step from the model. After recording the base line for 30 min, the animals were directly administered PB (0.026 mg/kg, im), followed by DFP (4.0 mg/kg, sc,) after 30 min and a mixture of atropine (0.2mg/kg) and 2-PAM (25 mg/kg) after 1 min. Our data shows that the DFP model of seizure/SE is very reproducible in the absence of lithium with animals surviving for more than 24 h (Fig. 4). However, the amplitude of the seizure spikes were albeit decresed in the absence of lithium. Thus, absence of lithium does not seem to negate the generation of seizure/SE in the currently developed DFP model of seizure/SE.

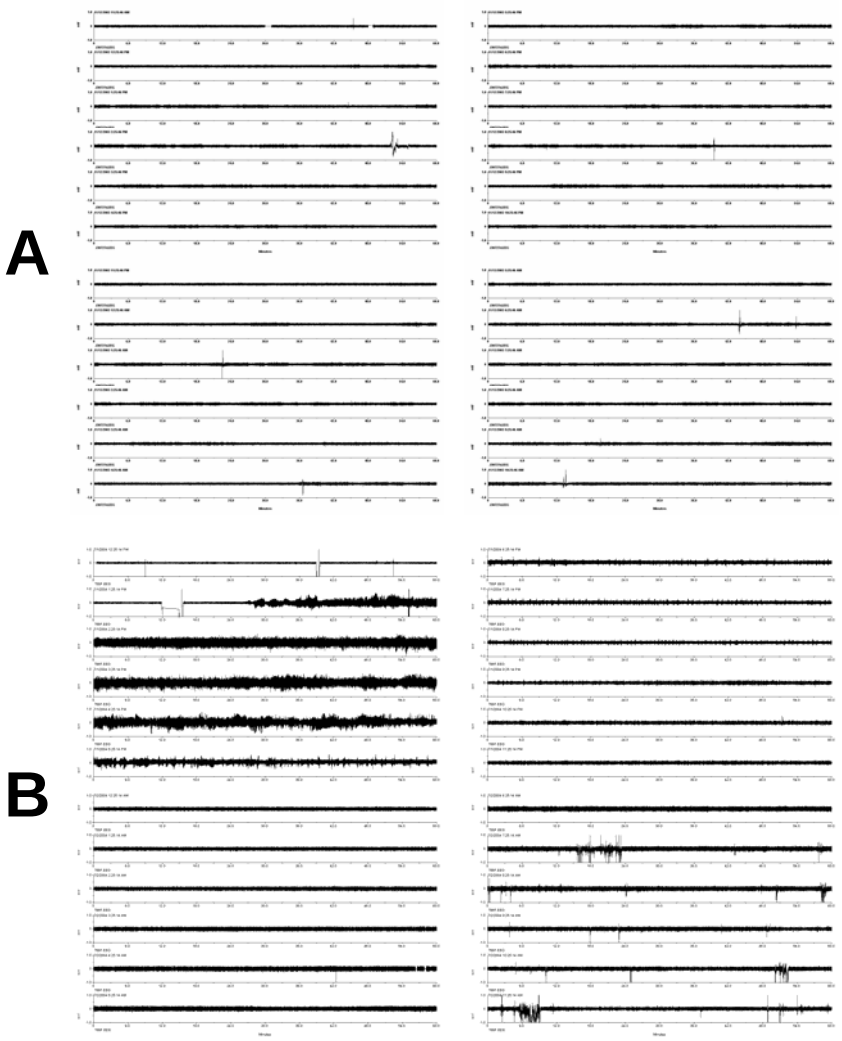


**Figure 4 : DFP model of seizure/SE in the absence of lithium chloride**
Base line EEG of rats implanted with telemetry probes were recorded for 30 min and then administered pyridostigmine bromide (0.026 mg/kg, im) followed by 300 ml saline and a mixture of atropine and 2-PAM (0.02 mg/kg and 25 mg/kg, im) was injected and the EEG was recorded. **B**. Rats were administered pyridostigmine bromide (0.026 mg/kg, im) followed by DFP (4 mg/kg, sc) freshly diluted in 300 ml phosphate buffered saline. One minute later a mixture of atropine and 2-PAM (0.2 mg/kg and 25 mg/kg, im) was injected, and the EEG was recorded continuously. The Y axis range was -1 to +1. Each line represents 1 h of EEG recordings. The DFP treated rat produced seizure/SE and survived more than 24 h. However, the amplitude of the seizure was lower than rats injected with LiCl (Fig. 3).

*Heart rate, body temperature, and physical activity in DFP treated animals.* In the current model, heart rate was slightly increased from the beginning level (440 beats/min) but within normal limits immediately after DFP treatment (Fig. 5A). However, with the increase in time the heart rate gradually decreased from the original level, and fell to 320 beats/min after 7 h in DFP treated animals. Exposure to DFP strongly reduced the body temperature from 36±1ºC to 30± 2ºC (Fig. 5B). The maximum reduction of body temperature was observed after 12 h of DFP exposure. The temperature gradually increased and tends to return normal at the end of 24 h. The heart rate gradually increased until the end of the experiment but did

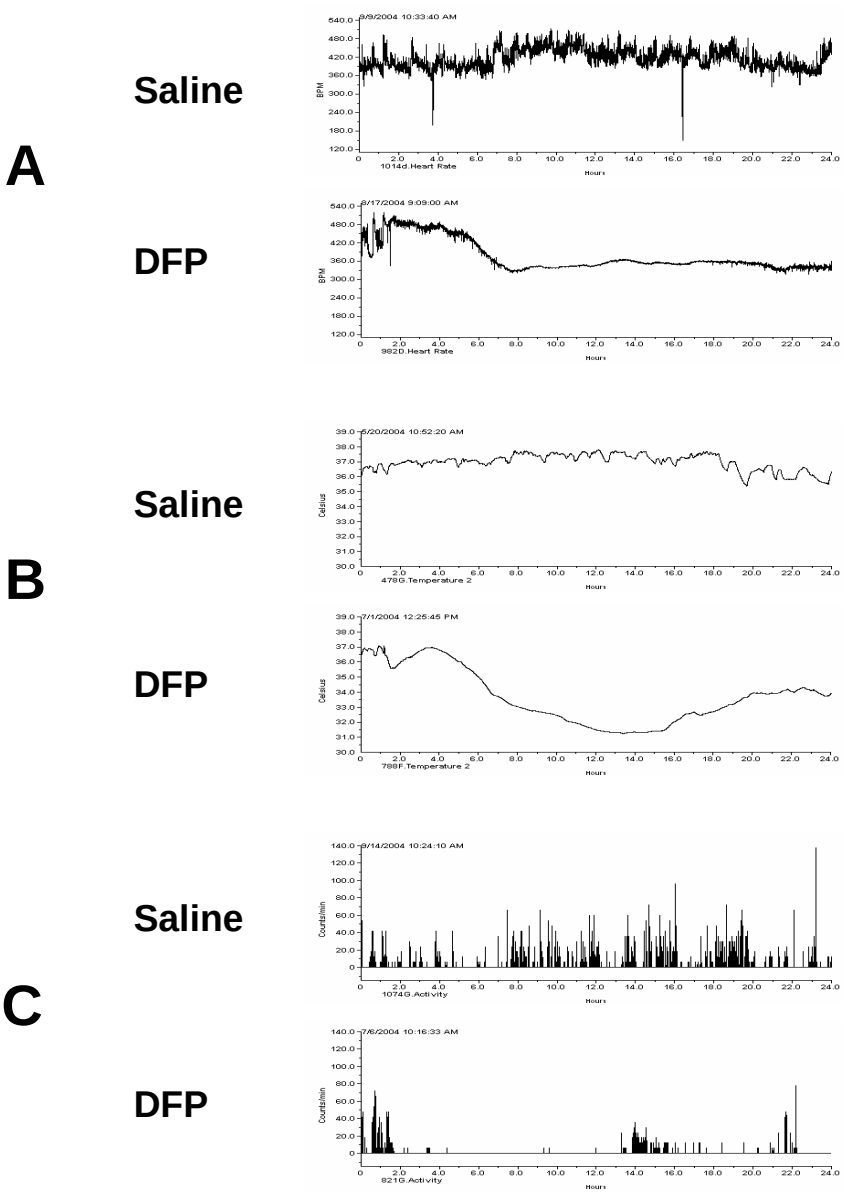


**Figure 5: Heart rate, body temperature, and physical activity in the DFP model of seizure/SE. A.** Rats implanted with telemetry probes were administered pyridostigmine bromide (0.026 mg/kg, im) followed by saline or DFP (4 mg/kg, sc) at 30 min and a mixture of atropine and 2-PAM (2 mg/kg and 25 mg/kg, im) was injected 1 min later. The heart rate was recorded continuously. The y-axis is beats/min, and the x-axis represents 24 h of recordings. Note that after DFP exposure the heart rate of DFP treated animal gradually increased from the beginning, though it is within the normal limits and then reduced. B. The body temperature was recorded continuously for 24 h. The y-axis is measured in degrees Celsius, and the x-axis represents 24 h of recording. Note that, temperature takes a quick dip after DFP exposure then becomes normal. Later the temperature gradually decreased and starts to become normal. C. Physical activity recordings of the saline and DFP treated animals. The y-axis is measured in counts/min, while the x-axis represents 24 h of recording. Note the activity of DFP treated animals was significantly reduced compared to the saline controls.

not return to baseline levels within the 24 h duration. Physical activity was reduced in DFP-treated animals compared to control saline treated animals (Fig. 5C). After an hour of DFP exposure the animal becomes quiet for a period of time. Such a period of quiet was also observed in animals exposed to soman. After the quiet period, the animals become gradually active.

*Behavioral defects in the DFP model of seizure/SE:* DFP exposure results in predominantly cholinergic symptoms with varieties of physical signs of intoxication and delayed neurotoxic effects (26;27). In the current DFP model, the first sign induced by DFP include chewing, followed by head tremors and jerky motion that became generalized to include the whole body. The animals showed Straub tail, gasping like movements and the seizure activity was marked by rhythmic movement of the ears, facial musculature, forepaw clonus, salivation. After 24 seizure/SE, the animals still exhibit several behavioral problems that are similar to soman exposed animals (Table 1) (28).

**TABLE 1.**

Neurobehavioral comparison of DFP treated rats Vs 1.6 $LD_{50}$ soman exposed rats

| Parameter | Soman | DFP |
|---|---|---|
| Sensitivity | Male | Male |
| Excessive salivation | 1-2 min after seizure | 1-2 min after seizure |
| Quiet | After 2-8 h | After 2 h |
| Voltage of discharge | Progressive decrease | Progressive decrease |
| Frequency | Eventually low | Eventually low |
| Low frequency spikes | Fade over time | Fade over time |
| Clonic jerks of the head & shoulder | Regular | Regular |
| Episodes of facial and forelimb clonus | Repetitive | Repetitive |
| Rearing on hind legs | Yes | Yes |
| Weight loss | Yes | Yes |
| Episodes Straub tail | Brief | Brief |
| Fasciculation of the back and trunk | Prominent | Prominent |
| Protrusion of the eye | No | Significant |
| Posture after 24 h | Hunched | Hunched |

*Neuropathology in the DFP model of seizure/SE*: Histopathology of the brain of the DFP treated animals that survive 24 h was analyzed in a blind fashion. DFP was capable of inducing neuropathology under the conditions of the seizure/SE model (Fig 6A). The lesion involves neuronal necrosis, consisting of shrunken pyknotic/necrotic individual neurons. Lesions were scored according to McDonough et al (29;30) and graded in a scale from 0-4 (Fig 6B). Table 2 displays the average brain lesion scores for individual brain regions in DFP exposed animals. Overall, the amygdala, cortex and thalamus showed the most frequent and severe damage.

**TABLE 2**

Neuronal damage in different areas of the brain following exposure to DFP

| Treatment | Neocortex | Hippocampus | Thalamus | Pyr. Cortex | Amygdala | Caudate |
|---|---|---|---|---|---|---|

| | | | | | | |
|---|---|---|---|---|---|---|
| Saline | 0 | 0 | 0 | 0 | 0 | 0 |
| DFP | 2.2 | 2 | 2.6 | 1.8 | 2.8 | 1.6 |

* No damage was found in cerebellum and brainstem of DFP treated rats. Grades are based on histologic analysis of H&E-stained brain sections, using the criteria of McDonough, et al, with slight modifications, as follows: 0 – no neuronal necrosis,1 – necrosis of 1-10% of neurons, 2 – necrosis of 11-25% of neurons, 3 – necrosis of 26-50% of neurons, 4 – necrosis of > 50% of neurons

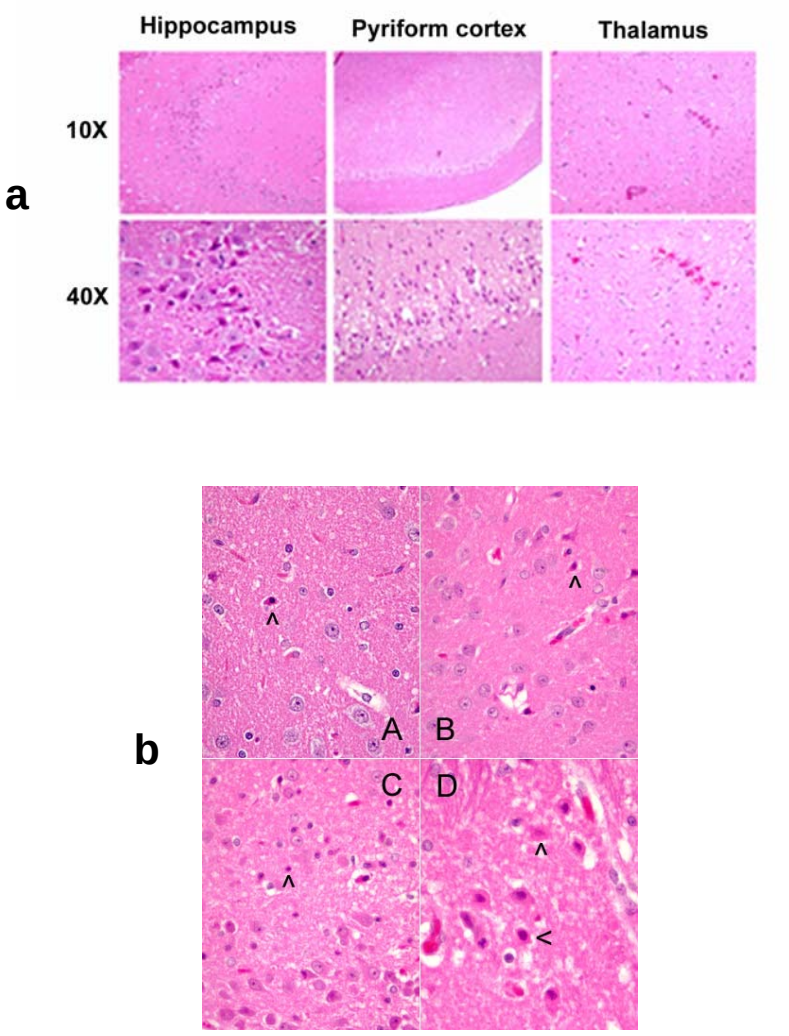


**Figure 6: Brain neuropathology in the DFP model of seizure/SE. a**. A histopathology figure showing the neuronal damage in DFP treated animals that produce seizure/SE and Survived more than 24 h. Most severe brain damage is represented by dark staining condensed chromatin. Note the argyrophilic darkly stained damaged neurons in DFP treated rats in the hippocampal region. The damage was severe in nucleus of the thalamus and pyriform cortex. Hippocampus also showed significant damage. There was no neuronal damage in control rats exposed to saline. **b**. Examples of different grades indicating neuronal necrosis in the thalamus. A = 1; B = 2; C = 3; D = 4. Some dead neurons in each image are indicated by arrowheads.

## DISCUSSION

The present study reveals the development of a rat seizure/SE model using a safer organophosphate, diisopropylflurophosphate. Previous studies indicated that DFP is unable to

produce seizure/SE similar to soman or paraoxon (31). DFP has been shown to produce short convulsive bursts that were frequently lethal. (32-34). The reason why DFP was unable to produce seizure could be due to the narrow dosage range between DFP and unlike other potent OPs. In the case of DFP, a minor change from the effective dose leads to increased mortality. Narrow range of DFP dose presents a problem to select a dose of DFP necessary to produce seizure/SE without mortality. This could be the reason why induction of a seizure/SE by DFP was not reported previously. We were able to determine the dose of DFP as well as atropine that induce seizure/Se in all of the animals tested with a survival period more than 24 h that will permit observation of the effects of pretreatment or post exposure treatment with neuroprotectants. In the current model increase in 0.5 mg/kg DFP from 4.00 mg/kg result in increased mortality. To our knowledge this is the first report that successfully describes a seizure/SE model using DFP that lasts for more than 24 h.

Although some studies used DFP induced seizure/SE for studying the mechanism of pathology and the evaluation of neuroprotectants, in most of these cases seizure was detected by motor convulsions (12;15;31). However, we found that lower doses of DFP treated animals showed strong convulsions although they did not induce any CNS seizure. This indicates that tonic –clonic seizures cannot be substituted for CNS depolarization and seizure. Recording the EEG by radiotelemetry serves an important role in the seizure/SE model. DFP did not produced prolonged convulsions even after lithium chloride pretreatment that reduce the threshold for seizure/SE rats. However, in the current DFP model of seizure/DE, lithium did not play a significant role in the successful model. All the animals seized to similar extent in the absence of lithium. Elimination of lithium in the DFP model of seizure/SE is important because lithium may activate signaling pathways and may cause several biochemical effects (31;32).

In the present study we found that the volume of the DFP administered is critical for the success of the seizure/SE model. Minimum volume of diluted DFP 200 microliter or more is optimal for inducing seizure/SE. Lower volumes of DFP was able to produce only low amplitude seizure/SE irrespective of the dose range. Increasing the dose of DFP under these conditions causes severe respiratory problems and eventually the animal dies within a short time after the onset of seizure/SE. It is possible that larger volume of DFP may help to equilibrate the DFP in the systemic circulation and the body. Quicker equilibration may exclude the possibility of localized DFP toxicity at the torso that may lead to cardiovascular problems and abrupt death.

Attempt to increase the survivability of animals after the onset of seizure/SE following DFP administration by pre-treatment with 1 mg/kg, sc, methyl atropine (a muscarinic antagonist that do not enter the brain) was not successful. Compared to DFP alone, pretreatment with methyl atropine showed that more DFP is required to induce seizure/SE in the presence of methyl atropine, probably due to the DFP mediated damage of the blood brain barrier and leakage of the drug to the brain. Present data show that the dose window of DFP and atropine has to be balanced for proper induction seizure/SE. Mere increase in atropine prevents seizure generation and is not sufficient to increase the survivability of animals seizing at higher dose of DFP that can induce seizure/SE.

Blood AChE levels were significantly more than 65% in DFP treated animals compared to controls. This is consistent with the fact that more than 65% inhibition of AChE activity is required to induce seizure/SE. Spinal cord AChE activity that is similar to Brain AChE was also significantly inhibited by DFP. Thus, a recovery in the Brain AChE activity could be used to evaluate the efficacy neuroprotects being studied for protection against OP exposure. Also the recombinant AChE or BChE can be evaluated for pre- or post- exposure treatment against OP exposure.

The DFP seizure model reported here is very reproducible and seems to be similar to chemical warfare nerve agent exposure in rats described by McDonough et al (30;35). The kinetics show that the seizure induction in the DFP model is at 8-10 min after the administration of DFP. Furthermore, neuropathology data show that similar to soman the highest lesion was in the amygdala brain region following exposure to DFP. Hippocampal region also showed significant neuronal

damage following DFP exposure. Recently, a similar kinetics and neuropathology by various chemical warfare nerve agents has been reported in a guinea pig model (16).

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## CONCLUSIONS

In summary, a DFP model of seizure/SE in which animals survives for more than 24 h has been developed in rats. The model is very similar to chemical warfare nerve agent exposure. This safe model will be very useful to investigate the mechanism of neuropathology following chemical warfare agents and screening of neuroprotectants. Currently we are studying the mechanism of neuropathology and screening adenosine receptor agonists using the DFP model of seizure/SE.

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